

Inhibition of Oxidative and Antioxidative Enzymes by Trans-Resveratrol

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ABSTRACT: Trans-resveratrol, a phytoalexin produced by a variety of plants, has been shown to inhibit oxidative enzymes in an animal cell system. Its effect on several oxidative and antioxidative enzymes from plants was investigated using *in vitro* assays. Trans-resveratrol inhibited superoxide dismutase, lipoxygenase, catalase, peroxidase, polyphenol oxidase, and 1-aminocyclopropane-1-carboxylic acid oxidase with apparent K_i 's of 10, 90, 100, 255, 305, and 350 μM , respectively. Trans-resveratrol inhibited lipoxygenase activity more effectively than other lipoxygenase inhibitors, including propyl gallate, ibuprofen, ursolic acid, acetylsalicylic acid, and salicylhydroxamic acid.

Key Words: trans-resveratrol, superoxide dismutase, lipoxygenase, catalase, peroxidase, polyphenol oxidase, and 1-aminocyclopropane-1-carboxylic acid oxidase.

Introduction

OXIDATIVE ENZYMES ARE OF INCREASING INTEREST TO THE plant scientist as well as the food industry because of their effects on plant metabolism and the nutritional benefits they impart to plant foods. Polyphenol oxidase (PPO) is responsible for enzymatic browning in a wide range of vegetable, fruit, and juices (Mayer and Harel 1979). Peroxidases may be responsible for the generation of free radicals, and may have a role in lignin synthesis, hormone metabolism, and response to stress (Gaspar and others 1982). Another enzyme, lipoxygenase, catalyzes the oxidation of polyunsaturated fatty acids to produce hydroperoxides which can be broken down by other enzymes (Siedow 1991). Lipoxygenase may also catalyze the oxidation of carotenoids, including β -carotene. Lipoxygenase has a profound influence on color, flavor, texture, and nutritive properties of food (Robinson and others 1995). Another important enzyme in plant systems, 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase, catalyzes the synthesis of the plant hormone ethylene (Yang and Hoffman 1984). Enzymes, such as catalase and superoxide dismutase (SOD), may serve to inactivate oxygen species and function as antioxidants in biological systems (Larson 1988).

Active oxygen species are generated during normal aerobic metabolism. These free radicals can cause deleterious oxidation of lipids, proteins and nucleic acids that seriously perturb normal cell metabolism. Plants and animals possess numerous antioxidant systems for protection against these active oxygen species. Trans-resveratrol, a natural compound, has been found in a variety of plants; for example, grapes and peanuts (Gorham 1980). Trans-resveratrol has been suggested to reduce heart disease and to have anti-cancer activity. These effects could be due to trans-resveratrol's ability to inhibit low-density lipoprotein oxidation (Frankel and others 1993), block platelet aggregation (Kimura and others 1985, Chung and others 1992) and eicosanoid synthesis (Pace-Asciak and others 1995). Trans-resveratrol also acts as an antioxidant and inhibits cyclooxygenase and hydroperoxidase activities in an animal cell system (Jang and others 1997). Trans-resveratrol is a phytoalexin produced during

environmental stress or pathogenic attack (Langcake and Pryce 1976). However, the physiological function of trans-resveratrol in plants is not well defined. In this study, the effect of trans-resveratrol on several oxidative and antioxidative enzymes found in plants was investigated.

Materials and Methods

Chemicals.

All chemicals were purchased from Sigma (St Louis, Mo., U.S.A.). A stock solution of trans-resveratrol (10 mM) was prepared in ethanol. Trans-resveratrol in the assay buffer was diluted from this stock solution. There were 3 replicates for all enzyme assays.

Preparation and assay of enzymes.

Lipoxygenase. Lipoxygenase from soybean was purchased from Sigma and assayed as described by the supplier, with modification using linoleic acid as the substrate. Activity was measured by incubating the enzyme solution with 17.8 μM linoleic acid in 0.2 M Na-borate (pH 9.0) containing 5% ethanol. The absorbance at 236 nm was monitored for 5 min with a spectrophotometer (HP 9451A, Hewlett Packard, Avondale, Pa., U.S.A.) at 22 °C. One unit of activity was defined as an absorbance increase of 0.001 per min. The effect of trans-resveratrol on lipoxygenase was compared with that of other lipoxygenase inhibitors, propyl gallate, ibuprofen, ursolic acid, acetylsalicylic acid, and salicylhydroxamic acid. All inhibitors were present in the assay at a concentration of 267 μM .

PPO. 'Fuji' apple peel (10g) was homogenized for 3 min with 50 ml cold acetone (-20 °C). The homogenate was filtered through a glass fiber filter (Whatman GF/C) in a Buchner funnel, and the residue washed with cold acetone until a colorless filtrate was obtained. The acetone powder was dried under vacuum. PPO was extracted by suspending 2 g of the acetone powder in 90 ml 50 mM Na-phosphate (pH 7.0) with 1 M KCl. After agitation on ice for 30 min, the mixture was filtered through Miracloth (Calbiochem, San Diego, Calif., U.S.A.), and centrifuged at 28,000g for 30 min. The super-

nant was used for further purification. A 30 to 80% $(\text{NH}_4)_2\text{SO}_4$ precipitation was dissolved in 20 ml 50 mM Na-phosphate buffer (pH 6.5), and dialyzed overnight against 0.4 M $(\text{NH}_4)_2\text{SO}_4$ and 0.4 M KCl at 4 °C. After centrifugation (28,000g, 10 min), the supernatant was purified by hydrophobic chromatography according to Trejo-Gonzalez and Soto-Valdez (1991). The supernatant was applied onto a Phenol Sepharose CL4B column (28 × 80 cm; Pharmacia, Piscataway, N.J., U.S.A.) that was equilibrated with a 50 mM Na-phosphate buffer (pH 6.8) with 0.4 M $(\text{NH}_4)_2\text{SO}_4$ and 0.1M KCl. PPO was eluted with a linear gradient of 0.4 M to 0 M $(\text{NH}_4)_2\text{SO}_4$ and 0.1 M to 0 M KCl at 1 ml·min⁻¹. All fractions (5 ml each) containing PPO activity were combined. PPO was assayed according to Sciancalepore and Longone (1984). The reaction mixture contained 1.5 ml 0.1 M Na-citrate (pH 5.0), 1 ml 0.02 M catechol in the same buffer, and 0.5 ml enzyme preparation. The increase in A_{420} was recorded every 10 sec up to 5 min from the time the enzyme extract was added. One unit of enzyme activity was defined as an absorbance increase of 0.001 per min at 22 °C.

Peroxidase. Peroxidase was extracted and purified similar to PPO. Total peroxidase activity was assayed by incubating 20 ml of extract with 1 ml of 100 mM guaiacol, 0.3 ml of 1% H₂O₂, and 1ml of 20 mM Na-phosphate buffer (pH 6.5) (Miller and others 1987). The reaction was initiated by the addition of H₂O₂ and the increase in A_{470} was determined for 5 min at 22 °C. One unit of enzyme activity was defined as an absorbance increase of 0.001 per min.

ACC oxidase. Extraction and assay of ACC oxidase from 'Fuji' apples were as described previously (Fan and others 1996). Briefly, cortex tissue was homogenized with 400 mM K-phosphate (pH 7.2) containing 10 mM NaS₂O₅, 4 mM 2-mercaptoethanol, and 3 mM Na-ascorbate. The homogenate was filtered and centrifuged at 28,000 g. The pellet was resuspended in 200 mM MOPS (pH 7.2), 1 mM dithiothreitol, 3 mM Na-ascorbate, 10% glycerol (W/V), and 0.2% Triton X-100 (V/V). The mixture was centrifuged and the supernatant was used to assay for ACC oxidase activity. The reaction mixture contained 100 mM MOPS (pH 7.2), 20 μM FeSO₄, 1mM

ACC, 1mM Na-ascorbate, 6% (V/V) CO₂ (in the gas phase) and 10% (W/V) glycerol in a sealed test tube. The reaction was initiated by addition of 100 μL enzyme extract. After 30 min incubation with shaking at 25 °C, headspace ethylene was measured by a gas chromatography.

SOD. SOD was extracted from 'Fuji' apples. Cortex tissue (20 g) was homogenized with 40 ml extraction buffer (0.1 M

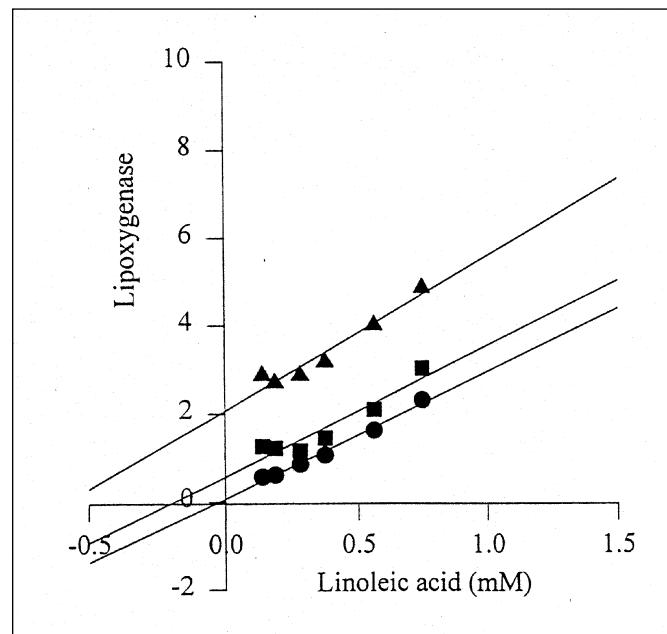


Figure 2—Lineweaver-Burk plot of lipoxygenase activity versus linoleic acid concentration at: o, 0; •, 100; △, 133 μM trans-resveratrol. Each data point is the mean of 3 replicates.

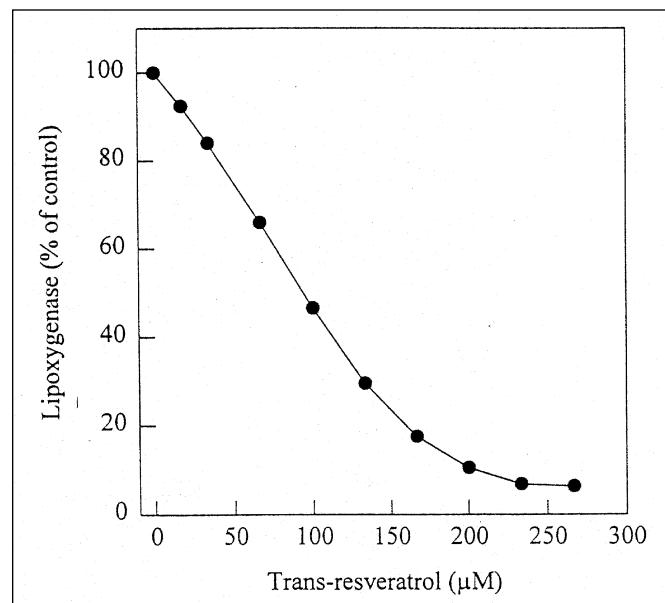


Figure 1—Effect of trans-resveratrol on lipoxygenase activity in vitro. Each data point is the mean of 3 replicates.

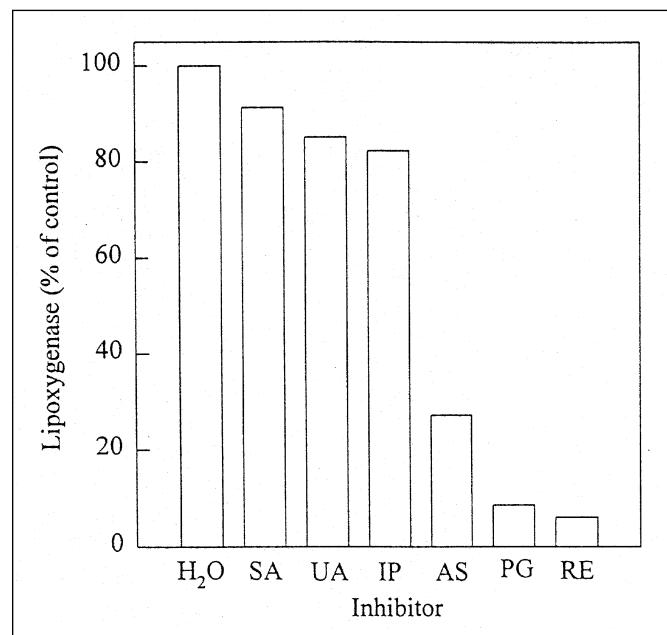


Figure 3—Comparison of trans-resveratrol (RE) inhibitory effect on lipoxygenase activity with propyl gallate (PG), ibuprofen (IP), ursolic acid (UA), acetylsalicylic acid (AS), and salicylhydroxamic acid (SA). All inhibitors were present in the assay buffer at 267 μM . Each data point is the mean of 3 replicates.

K-phosphate pH 7.8, 0.1 mM EDTA, and 1% PVPP for 5 min. The homogenate was filtered through Miracloth (Calbiochem, San Diego, Calif., U.S.A.), then centrifuged for 10 min at 12,000 g. The supernatant was used for SOD assays according to Giannopolitis and Ries (1977). The reaction mixture included 1 ml water, 1 ml of 0.05 M Na₂-CO₃ (pH 10.2) buffer containing 13 mM methionine, 1.3 μ M riboflavin and 63 μ M nitro blue tetrazolium, and 1 ml enzyme extract in a final volume of 3 ml. An identical tube without the enzyme extract served as a blank. Reactions were carried out for 30 min under illumination at 22 °C. The A₅₆₀ nm was measured and 1 unit of SOD activity was defined as the amount of enzyme which inhibited the reaction rate by 50%.

Catalase. Catalase was purchased from Sigma and assayed as described by the supplier. The reaction mixture contained 25 mM K-phosphate (pH 6.8) and 10 mM H₂O₂. Absorbance at 240 nm was monitored for 1 min after addi-

tion of enzyme at 22 °C. One unit was defined as the amount of enzyme decomposing 1 μ M H₂O₂ per min.

Results and Discussion

TRANS-RESVERATROL INHIBITED LIPOXYGENASE IN A CONCENTRATION-dependent manner, inhibition increased with trans-resveratrol concentration (Figure 1). At 90 μ M trans-resveratrol, lipoxygenase activity was half that of the controls. The inhibition of lipoxygenase activity by trans-resveratrol appeared to be uncompetitive with respect to linoleic acid (Figure 2). Trans-resveratrol was the most effective

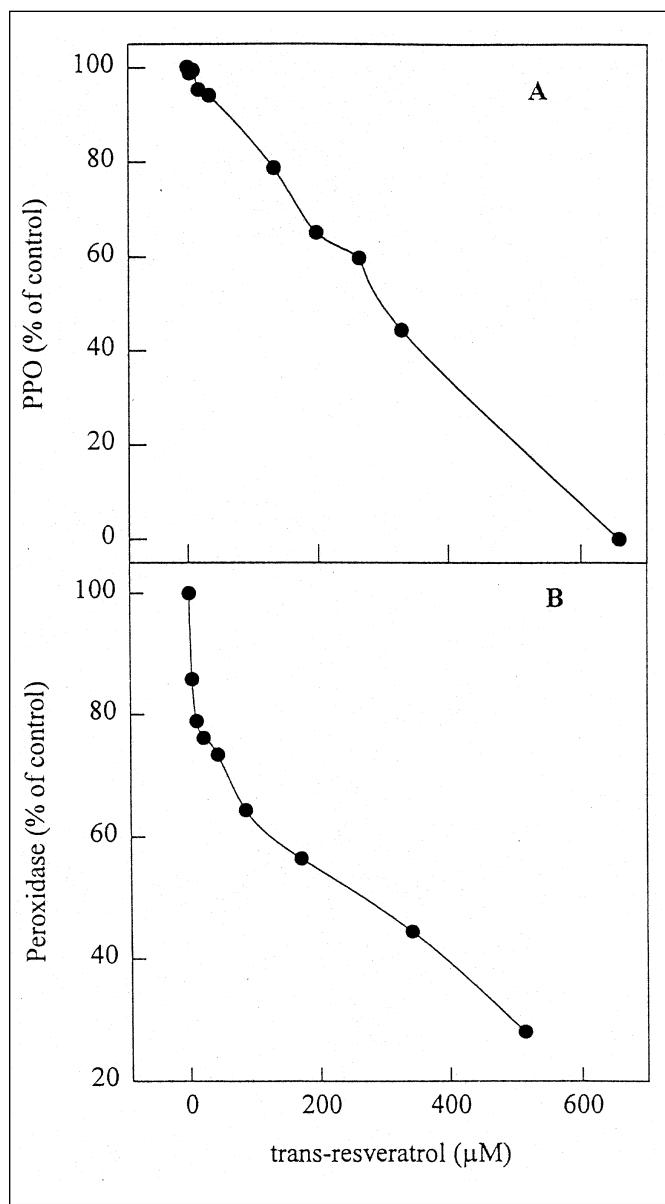


Figure 4—Effect of trans-resveratrol on polyphenol oxidase (PPO) (A) and peroxidase (B) activity in vitro. Each data point is the mean of 3 replicates.

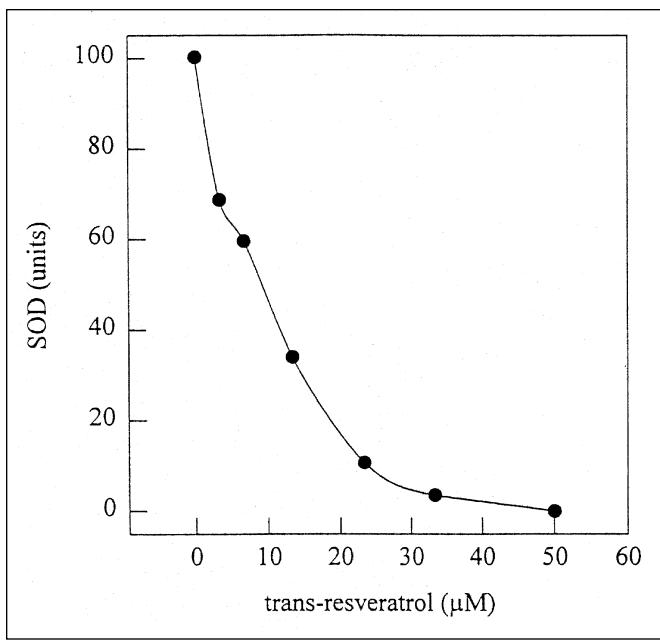


Figure 5—Effect of trans-resveratrol on superoxide dismutase (SOD) activity in vitro. Each data point is the mean of 3 replicates.

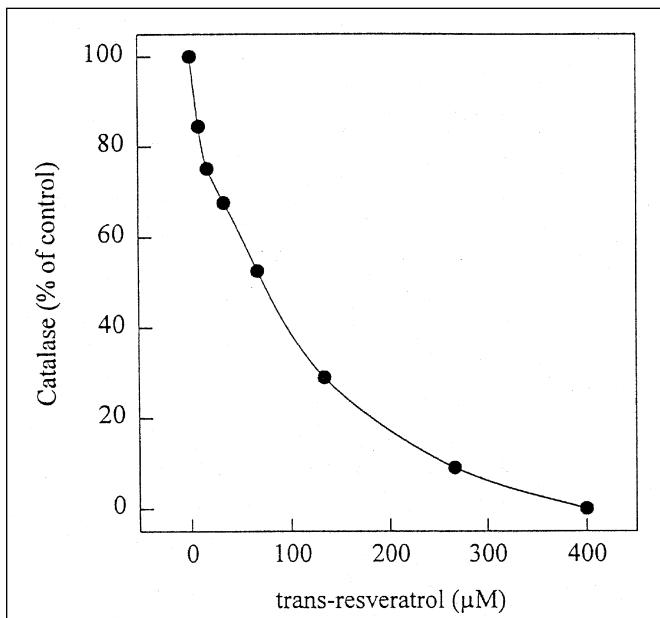


Figure 6—Effect of trans-resveratrol on catalase activity in vitro. Each data point is the mean of 3 replicates.

among the lipoxygenase inhibitors tested (Figure 3).

Trans-resveratrol inhibited PPO (Figure 4A) and peroxidase (Figure 4B) with apparent K_i s of 305 μM and 255 μM , respectively. The relationship between PPO activity and concentration of trans-resveratrol appeared to be linear. SOD was inhibited by trans-resveratrol with an apparent K_i of only 10 μM (Figure 5). Catalase was inhibited by trans-resveratrol with a K_i of 100 μM , but activity was detectable even at high trans-resveratrol concentrations (Figure 6). ACC oxidase was also inhibited by trans-resveratrol with an apparent K_i of 350 μM , but the enzyme was not totally inhibited even at 3.2 mM (Figure 7).

Many oxidase enzymes require a metal co-factor which is believed to undergo redox changes during the course of catalysis. Lipoxygenase is an Fe containing dioxygenase (Siedow 1991), and there are 3 types of SOD classified by their metal cofactor: Cu/Zn, Mn or Fe (Bannister and others 1987). ACC oxidase requires Fe as a cofactor (Smith and others 1992). PPO may have Cu (Mayer and Harel 1979) and peroxidase uses Fe as cofactors (Gaspar and others 1982). Trans-resveratrol is a phenolic compound containing 2 -OH groups. Any molecule with an unshared electron pair can coordinate to form complexes with metal ions. Trans-resveratrol may act as a metal chelator and eliminate the availability of metal to participate in enzyme activity.

Trans-resveratrol may also serve as a substrate for the oxidase enzymes. Peroxidase is capable of oxidizing trans-resveratrol and forms a compound with a light brown color (Morales and others 1997). Lipoxygenase may also use trans-resveratrol as a substrate as a light-brown compound formed during our assays (data not shown). Trans-resveratrol may also form a complex with the lipoxygenase substrate, linoleic acid, in the absence of lipoxygenase. We ob-

served formation of a white precipitate upon the addition of trans-resveratrol to the linoleic acid solution. Trans-resveratrol is a phenolic compound and many phenolics have been shown to inhibit lipoxygenase (Richard-Forget and others 1995). PPO catalyzes oxidation of phenolic compounds and could possibly use trans-resveratrol as a substrate. Trans-resveratrol accumulates in response to fungal infection, UV light, or wounding, and has microcidal activity (Langcake and Pryce 1976; Creasy and Coffee 1988). This activity may be due to of its strong inhibition of oxidative enzymes at very low concentrations. The strong inhibition of oxidases by trans-resveratrol may prove to be a useful tool for the food industry.

References

- Bannister JV, Bannister WH, Rotilio G. 1987. Aspects of the structure, function and application of superoxide dismutase. CRC Crit Rev Biochem. 22:111-180.
- Chung MI, Teng CM, Cheng KL, Ko FN, Lin CN. 1992. An antiplatelet principle of *Veratrum formosanum*. Planta Med 58:274-76.
- Creasy LL, Coffee M. 1988. Phytoalexin production potential of grape berries. J Amer Soc Hort Sci 113:230-234.
- Fan X, Mattheis JP, Fellman JK. 1996. Inhibition of apple fruit 1-aminocyclopropane-1-carboxylic acid oxidase activity and respiration by acetylsalicylic acid. J Plant Physiol 14:469-471.
- Frankel EN, Waterhouse AL, Kinsella JE. 1993. Inhibition of human LDL oxidation by resveratrol. Lancet 341:1103-1104.
- Gaspar TH, Penel CL, Thorpe T, Greppin H. 1982. Peroxidases 1970-1980: a survey of their biochemical and physiological roles in higher plants. 324 p. Geneva: Univ. of Geneva Press.
- Giannopolitis CN, Ries SK. 1977. Superoxide dismutase. I. Occurrence in higher plants. Plant Physiol 59:309-314.
- Gorham J. 1980. The stilbenoids. In Reinhold L, Harborne JB, Swain T, editors. Progress in Phytochemistry. Oxford: Pergamon Press. pp. 203-252.
- Jang M, Cai L, Udeani G, Slowing K, Thomas C, Beecher C, Fong H, Farnsworth N, Kinghorn A, Mehta R, Moon R, Pezzuto J. 1997. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. Science 275:218-220.
- Kimura Y, Okuda H, Arichi S. 1985. Effects of stilbene on arachidonate metabolism in leukocytes. Biochem Biophys Acta 834:275-278.
- Langcake P, Pryce RJ. 1976. The production of resveratrol by *Vitis vinifera* and other members of the Vitaceae as a response to infection or injury. Physiol Plant Pathol 9:77-86.
- Larson RA. 1988. The antioxidants of higher plants. Phytochem 27:969-978.
- Mayer AM, Harel E. 1979. Polyphenol oxidases in plants. Phytochem 18:193-215.
- Miller AR, Dalmasso JP, Kretchman DW. 1987. Mechanical stress, storage time, and temperature influence cell wall-degrading enzymes, firmness and ethylene production by cucumbers. J Amer Soc Hort Sci 112:666-671.
- Morales M, Alcantara J, Ros Barcelo A. 1997. Oxidation of trans-resveratrol by hypodermal peroxidase isoenzyme from Gamay rouge grape (*vitis vinifera*) berries. Am J Enol Vitic 48:33-38.
- Pace-Asciak CR, Hahn SE, Diamandis EP, Soleas G, Goldberg DM. 1995. The red wine phenolics trans-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: Implications for protection against coronary heart disease. Clin Chem Acta 235:197-219.
- Richard-Forget F, Gauillard F, Hugues M, Jean-Marc T, Boivin P, Nicolas J. 1995. Inhibition of horse bean and germinated barley lipoxygenases by some phenolic compounds. J Food Sci 60:1325-1329.
- Robinson DS, Wu Z, Domoney C, Casey R. 1995. Lipoxygenase and the quality of foods. Food Chem 54:33-43.
- Sciancalepore V, Longone V. 1984. Polyphenol oxidase activity and browning in green olives. J Agric Food Chem 32:320-321.
- Siedow JN. 1991. Plant lipoxygenase: structure and function. Ann Rev Plant Physiol Plant Mol Biol 42:145-188.
- Smith JJ, Vergeridus P, John P. 1992. Characterization of the ethylene-forming enzyme partially purified from melon. Phytochem 31:1485-1494.
- Trejo-Gonzalez A, Soto-Valdez H. 1991. Partial characterization of polyphenol oxidase extracted from 'Anna' apple. J Amer Soc Hort Sci 116(4):672-675.
- Yang SF, Hoffman NE. 1984. Ethylene biosynthesis and its regulation in higher plants. Ann Rev Plant Physiol 35:155-189.
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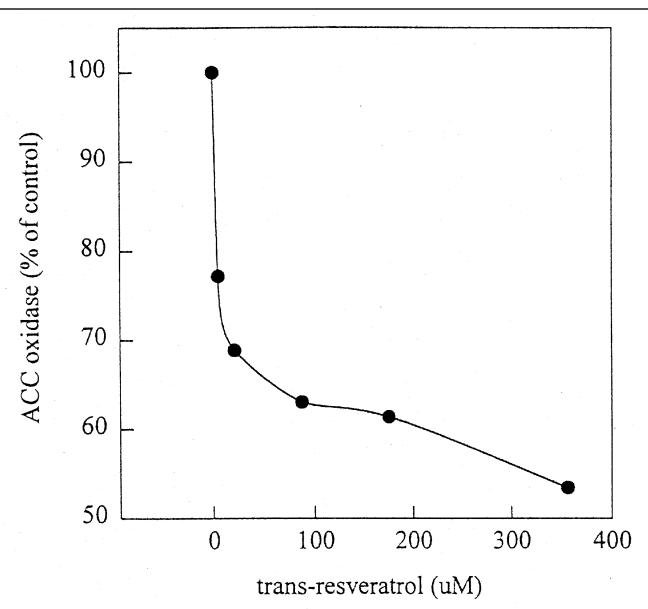


Figure 7—Effect of trans-resveratrol on 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase activity in vitro. Each data point is the mean of 3 replicates.